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# CHEMICAL ANALYSES OF ANTERIOR AND POSTERIOR BLASTOMERES OF CIONA INTESTINALIS

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Segregations of specific cytoplasms occur during early cleavage of the ascidian egg (Conklin, 1905) of which the most obvious is the localization of the myoplasm, presumptive for larval musculature, in the posterior cells at the four-cell stage. Cytological (Meves, 1913; Duesberg, 1915; Conklin, 1931) and cytochemical (Ries, 1937) studies indicate the localization of granules, presumably mitochondria, within the myoplasm. Recently Reverberi (1956) followed the distribution of mitochondria during development, using the vital stain, Janus green. The quantitative measurements of cytochrome oxidase in anterior and posterior blastomeres (Berg, 1956) gave a biochemical confirmation of the above studies as regards the localization of mitochondria.

The present study is a continuation of quantitative chemical analyses of anterior and posterior blastomeres from the four-cell stage of *Ciona,* choosing constituents which might be expected to be located on cellular particles. The minute amounts of cytoplasm available require the use of microchemical methods and limit the number of substances which may be studied; however, differences in activities of succinic dehydrogenase, apyrase, acid phosphatase, and ribonucleic acid have been found in homogenates of the two types of cells.

## Methods

Chorions were digested off unfertilized *Ciona* eggs in 3 per cent protease in sea water. The "naked" eggs were washed thoroughly, fertilized, and transferred to agar-coated dishes. At the end of the first cleavage the blastomeres were separated in large numbers by agitation and as each of these cleaved in turn, the anterior and posterior cells were separated with the tip of a fine braking pipette and segregated into different dishes.

The posterior blastomere is recognizable by an elongated shape and a clear cytoplasmic cap (Castle, 1896). These characteristics are transitory and the period for separation is critical; separation before completion of the cleavage furrow results in cytolysis whereas shortly after cleavage identification becomes increasingly difficult due to sphering of the posterior cell. This period was extended by lowering the temperature to 8–10° C. after completion of cleavage, thus prolonging the elongated state of the posterior cell. Furthermore it was discovered that with oblique

lighting the posterior cell, even after sphering, exhibits a bright crescentic rim of cytoplasm which is the remnant of the clear cytoplasmic cap. This persists for some minutes after cleavage and greatly extends the length of time during which identification is possible.

Separation of blastomeres was begun as soon as possible after cleavage, using the difference in shapes for identification. As the posterior cells began to round up, an oblique lighting was adopted and separation continued, using the bright rim of the posterior cell as a marker. In this manner several hundred blastomeres could be isolated, a considerable improvement over initial attempts where a maximum of 30–40 were obtained (Berg, 1956).

The desired number of segregated blastomeres was counted and transferred to 0.1-ml. centrifuge tubes by means of a braking pipette. After light centrifugation excess sea water was removed, a few $\mu$l. of homogenizing solution were added and the cells homogenized by drawing them in and out of a fine-bore pipette. The homogenization was carried out at 1–2° C., attained by placing the centrifuge tube in a previously chilled copper block. All micromethods were spectrophotometric, using the Beckman spectrophotometer adapted for the use of microcuvettes (Lowry and Bessey, 1946).

Succinic dehydrogenase activity in homogenates was measured by the method of Cooperstein *et al.* (1950). The blastomeres were homogenized in 1 $\mu$l. of 0.19 *M* sodium succinate; 5 $\mu$l. of 0.19 *M* succinate and 2 $\mu$l. of 0.03 *M* sodium cyanide were added and the mixture transferred to 45 $\mu$l. of $2 \times 10^{-5}$ cytochrome *c* contained in a microcuvette. All solutions were buffered with 0.04 *M* phosphate buffer at pH 7.4. The reduction of cytochrome *c* was followed at 550 m$\mu$ for three minutes at the end of which time a few grains of sodium hydrosulfite were added to completely reduce the cytochrome *c*. A semi-logarithmic plot of the readings, after substraction of the optical density of reduced cytochrome *c*, gave a straight line from which a velocity constant, ($\Delta$log ferricytochrome $c/\Delta T$), could be calculated. Succinic dehydrogenase activities were expressed as velocity constants for rates of reduction of cytochrome *c*.

Acid and alkaline phosphatases and apyrase were measured by micromethods described by Lowry *et al.* (1954). For acid phosphatase 10 $\mu$l. of substrate (8 mM disodium p-nitrophenyl phosphate in 0.05 *M* succinate buffer, pH 5) were mixed with the homogenate. After thirty minutes of incubation at 25° C., 45 $\mu$l. of 0.1 N NaOH were added, with immediate mixing, and read at 410 m$\mu$ within thirty minutes.

Alkaline phosphatase was measured in a similar manner except that a buffer (2 amino-2 methyl-1-propanol) at pH 10 was used with an incubation period of one hour. It was not necessary to carry out protein precipitations in determinations of either acid or alkaline phosphatase. Blanks were prepared by separate incubation of substrate and homogenate with mixing just before addition of NaOH.

Apyrase measurements were made by homogenizing the cells in 0.75 per cent sodium desoxycholate, a procedure which considerably increases the enzyme activity presumably due to particle breakdown. Ten $\mu$l. of substrate (2.5 mM adenosinetriphosphate in 0.05 *M* tris hydroxy-amino methane at pH 8.0) were added to the homogenate and the mixture incubated for one hour at 25° C. Protein was precipitated by adding 2 $\mu$l. of 30 per cent trichloracetic acid and, after centrifugation, the supernatant was transferred to another tube with 100 $\mu$l. of molybdate-ascorbic

acid reagent and read at 870 mμ. Blanks were prepared by separate incubation of substrate and homogenate with mixing at the time of addition of trichloracetic acid. Both apyrase and acid phosphatase activities were expressed as optical densities after subtraction of blank values.

Ribonucleic acid was determined by a micromethod based on the procedure of Ogur and Rosen (1950). Cells were extracted with 60 μl. of cold 70 per cent alcohol for 5–10 minutes followed by extraction with 60 μl. of warm alcohol-ether (3 : 1). After a few minutes extraction with cold 0.1 *M* perchloric acid, a final extraction with 45 μl. of 1.0 *M* perchloric acid was carried out for 48 hours.

A typical absorption curve for ribonucleic acid was obtained with the latter extract and the optical density at 260 mμ was used as a measure of the amount. Repeated test extractions with 70 per cent alcohol, alcohol-ether, and 0.1 *M* perchloric acid demonstrated that these solutions removed, within a few minutes, all amino acids, polypeptides and acid-soluble substances which absorb at 260 mμ. Although most extractions were carried out for a longer time, nearly all the ribonucleic acid was removed within 24 hours.

Protein was measured by the method of Lowry *et al.* (1951). The cells were placed in a 0.5-ml. test tube and 100 μl. of alkaline copper solution added and mixed. After 10 minutes, 10 μl. of diluted Folin reagent were added with immediate mixing and the sample read at 750 mμ. Addition of the Folin reagent is critical and the reliability of color development depends upon the rapidity and effectiveness of the mixing. On a micro scale this is difficult to control and the resulting variability seriously limits this method.

Calibration curves (Fig. 1) were made for each of the micromethods. Fertilized eggs, with the chorions removed, were used for these tests and as shown in Figure 1 the optical density measurements, or velocity constants for succinic dehydrogenase, are proportional to enzyme activities as represented by the number of eggs. The optical densities at 260 mμ of perchloric acid extracts, representing amounts of ribonucleic acid, are proportional to the number of eggs extracted.

## Results

Anterior and posterior blastomeres of *Ciona* were separated and kept at 1° C. in agar-coated dishes until ready for counting and transference to the reaction tubes. From the calibration curves (Fig. 1) an estimation was made of the number of blastomeres necessary in each test for reliable measurements of the constituent. Thirty to forty cells in each tube were sufficient for enzyme and protein measurements, whereas nearly one hundred were required for a reliable measure of ribonucleic acid.

All determinations of enzymes, ribonucleic acid, and proteins in anterior and posterior blastomeres were paired; *i.e.*, two reaction tubes were used, one containing anterior cells, the other containing an equal number of posterior cells. The analyses were then carried out simultaneously. Due to the variability of results obtained with microchemical methods it was necessary to repeat the analyses a number of times and, as each experiment was on eggs from different animals and carried out under slightly different conditions, the data are expressed as ratios of activities or concentrations for each paired experiment. The average of these ratios is used to summarize the results although statistical significance was calculated directly from the paired series.

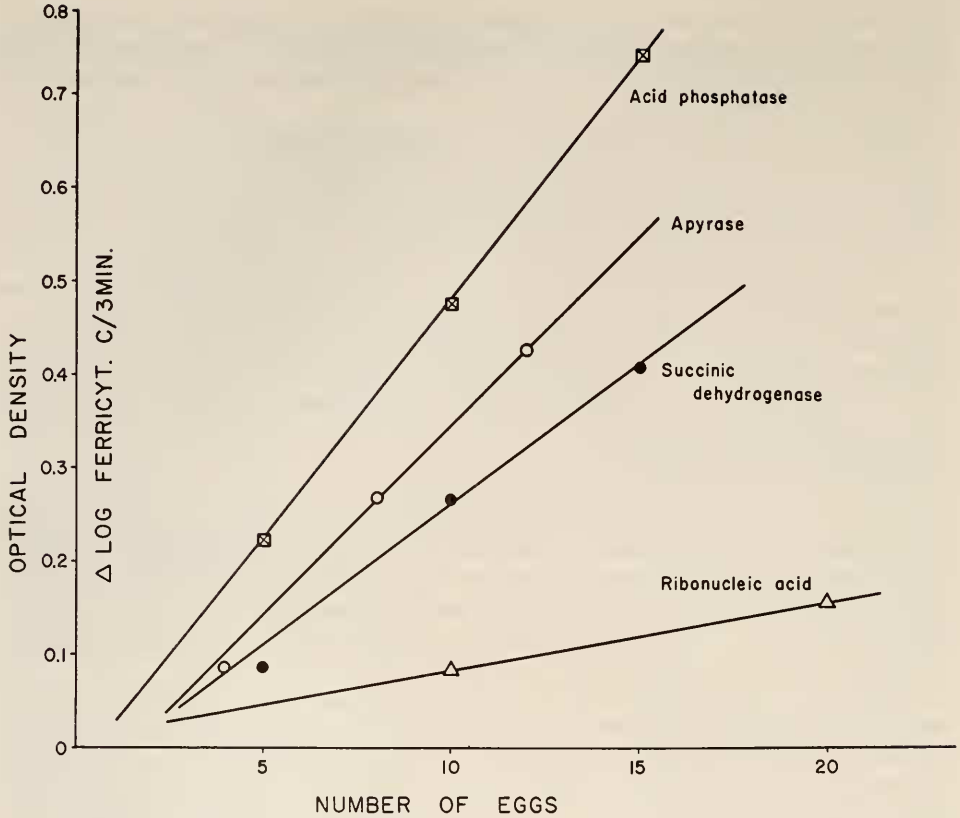


FIGURE 1. Acid phosphatase (activity expressed as optical density), apyrase (optical density), succinic dehydrogenase (Δ log ferricytochrome *c*/3 minutes) and ribonucleic acid (optical density) in homogenates of *Ciona* eggs.

The data for eighteen paired measurements of acid phosphatase in anterior and posterior cells are summarized in Table I. The anterior cells contain 12 per cent more of this enzyme than posterior cells, a difference which is significant at the one per cent level. A number of these experiments were carried out using sodium desoxycholate in the homogenization medium, with no detectable increase in enzyme activity.

TABLE I

*Acid phosphatase, expressed as optical densities, in homogenates of anterior and posterior cells of Ciona*

| Number of tests | Acid phosphatase in anterior cells | Acid phosphatase in posterior cells | Average ratio of paired determinations (ant./post.) |
|---|---|---|---|
| 18 (25–40 cells for each test) | 0.318 | 0.287 | 1.12 ± 0.03 |

A homogenate of thirty or more *Ciona* eggs was necessary to obtain even a detectable activity of alkaline phosphatase and accordingly it was not feasible to measure the activity of this enzyme in isolated blastomeres. The very low alkaline phosphatase activity in early cleavage stages is also characteristic of the mollusk *Mytilus edulis* and the sea urchin *Strongylocentrotus purpuratus.*

TABLE II

*Succinic dehydrogenase, expressed as velocity constants for reduction of cytochrome c, in homogenates of anterior and posterior blastomeres*

| Number of cells | Succinic dehydrogenase in anterior cells | Succinic dehydrogenase in posterior cells | Ratio (ant./post.) |
|---|---|---|---|
| 45 | 0.048 | 0.071 | 0.68 |
| 40 | .033 | .062 | .53 |
| 45 | .025 | .070 | .36 |
| 40 | .043 | .071 | .61 |
| 45 | .042 | .089 | .47 |
| | | Average | 0.53 ± 0.05 |

As would be expected on the basis of earlier measurements of cytochrome oxidase (Berg, 1956), succinic dehydrogenase activity is greater in posterior cells. The average of five experiments shows that homogenates of posterior cells contain twice as much of this enzyme as anterior cells (Table II).

TABLE III

*Apyrase, expressed as optical densities, in homogenates of anterior and posterior blastomeres*

| Number of blastomeres | Apyrase in anterior cells | Apyrase in posterior cells | Ratio (ant./post.) |
|---|---|---|---|
| 44 | 0.286 | 0.342 | 0.84 |
| 66 | .334 | .451 | .74 |
| 50 | .164 | .260 | .63 |
| 60 | .230 | .352 | .65 |
| 30 | .229 | .342 | .67 |
| 30 | .269 | .328 | .82 |
| 30 | .269 | .412 | .65 |
| 30 | .169 | .240 | .70 |
| 40 | .282 | .471 | .60 |
| | | Average | 0.70 ± 0.03 |

Apyrase measurements, summarized in Table III, show that the activity of this enzyme in homogenates of anterior cells is 70 per cent that in homogenates of posterior cells. In adult tissues (Frank *et al.,* 1950) and in amphibian embryos (Barth and Jaeger, 1947) apyrases with different pH activity characteristics have been extracted and thus for quantitative measurements of apyrases from different sources

it is necessary to determine pH-activity curves. Accordingly an attempt was made to determine the effect of pH on apyrases from anterior and posterior cells.

Succinate, tris-maleate, and ammediol buffers were used to cover the pH range from 5 to 9.3; pH values were checked with a glass electrode on mixtures prepared on a macroscale exactly as used for microanalyses. Apyrase activities were determined simultaneously at four different pH's in a paired series with thirty anterior or posterior blastomeres in each reaction tube. It was not feasible to cover the entire pH range in any one test; accordingly it was necessary to overlap pH values in

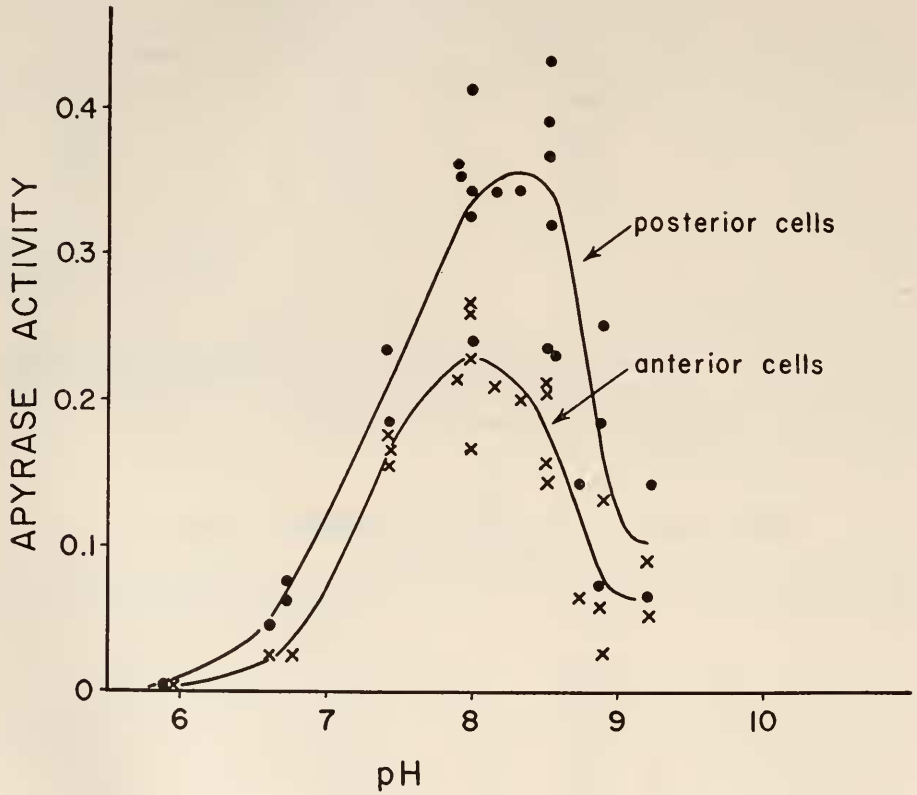


FIGURE 2. Apyrases in homogenates of anterior and posterior blastomeres; pH activity curves.

different experiments. The considerable variability of determinations was probably in part due to the necessity of constructing the curves from data obtained on different batches of eggs.

The pH-activity curves of apyrases from anterior and posterior cells (Fig. 2) are very similar although the optimum for posterior cell apyrase seems more alkaline. The difficulties of obtaining a pH-activity curve on such minute amounts of enzyme are so considerable that this slight difference cannot be considered significant.

The data for paired ribonucleic acid extractions, listed in Table IV, indicate that anterior cells contain 9 per cent less ribonucleic acid than posterior cells, a difference

which is significant. This was at first thought to be due to a slight volume difference between anterior and posterior cells. Visual observation of the four-cell stage often gives the impression that the posterior cells are larger. However, careful measurements of volumes by different methods failed to show any significant difference in size.

Diameters of blastomeres, with subsequent cleavages prevented by KCN or fixation in trichloracetic acid, were measured with an ocular micrometer. The average volume of 85 anterior blastomeres, as calculated from diameter measurements with cleavage inhibited by $3 \times 10^{-4}$ *M* KCN, was 97 per cent that of 75 posterior cells. The average volume of 134 anterior cells, after fixation in 2 per cent trichloracetic acid, was 98 per cent that of 178 posterior cells. Although with both types of measurements the average volume of the anterior cells was slightly less, statistical analyses of the data failed to show significant differences of these values.

TABLE IV

*Ribonucleic acid, expressed as optical densities at 260 mμ, extracted from anterior and posterior blastomeres*

| Number of blastomeres | RNA in anterior cells | RNA in posterior cells | Ratio (ant./post.) |
|---|---|---|---|
| 130 | 0.285 | 0.308 | 0.93 |
| 140 | .230 | .272 | .85 |
| 114 | .216 | .240 | .90 |
| 94 | .202 | .234 | .86 |
| 112 | .302 | .301 | 1.00 |
| 64 | .160 | .170 | .94 |
| 70 | .154 | .200 | .77 |
| 130 | .268 | .295 | .91 |
| 75 | .221 | .215 | 1.03 |
| 100 | .140 | .146 | .96 |
| 120 | .271 | .320 | .85 |
| 100 | .237 | .252 | .94 |
| | | Average | 0.91 ± 0.02 |

An additional determination of volumes was made indirectly by measuring relative amounts of protein in the two types of cells using a micromethod (Lowry *et al.*, 1951). Twenty-four paired measurements of protein, using 15–40 cells in each test, gave an average protein ratio (ant./post.) of 0.99 ± 0.046. The difference in ribonucleic acid content thus cannot be due to a volume difference and must represent a slight localization of this constituent.

## DISCUSSION

A localization of indophenol blue oxidase, presumably cytochrome oxidase, and succinic dehydrogenase in the ascidian embryo has been demonstrated by cytochemical methods (Ries, 1937; Reverberi and Pitotti, 1939; Mancuso, 1952). The microchemical measurements of cytochrome oxidase (Berg, 1956) and those of succinic dehydrogenase in the present study confirm the above cytochemical observations as to the localization of these enzymes in an early cleavage stage.

Information on the intracellular localization of enzymes in the ascidian egg is lacking; however a possible interpretation of the distribution of cytochrome oxidase, succinic dehydrogenase, and apyrase is that they are localized in mitochondria which are unequally distributed in the early cleavage blastomeres. There is considerable evidence that these enzymes are intracellularly located in mitochondria of a variety of cells (reviewed by Schneider and Hogeboom, 1951, 1956). A localization of mitochondria in the myoplasm of the ascidian egg has been demonstrated by cytological methods (Meves, 1913; Duesberg, 1915) and more recently by vital staining with Janus green (Reverberi, 1956). Reverberi (1957a) also presents evidence that cytochrome oxidase is intracellularly localized in the Janus green-staining mitochondria.

It is doubtful that the granules described by Duesberg (1915) and Conklin (1931), classified by them as mitochondria according to morphological and staining criteria, are responsible for the unequal distribution of enzymes. The granules in *Ciona* and "yellow mitochondria" in *Styela* are displaced by centrifugation to the centripetal pole (Conklin, 1931) whereas in centrifuged eggs the oxidase reactions are lacking in this region (Ries, 1939). The yellow granules in *Styela* may be displaced by light centrifugation without altering the localization of the oxidase reactions (Ries, 1942). In centrifuged homogenates of *Ciona* eggs cytochrome oxidase is found in the heavier fraction (Berg, 1956). Furthermore Reverberi (1957a) describes rod-like mitochondria, stainable with Janus green, which, in the centrifuged egg, collect at a different location than the granules described by Duesberg and Conklin. It appears probable, as Reverberi (1957a) also suggests, that several types of mitochondria are localized in the myoplasm of the ascidian embryo.

The intracellular localization of apyrase is probably to some extent within mitochondria, as has been shown for other types of cells (Schneider and Hogeboom, 1951, 1956), and the higher apyrase content of posterior cells most likely results from the segregation of mitochondria into these cells. The average ratio of activities in the two types of cells differs significantly from those for cytochrome oxidase or succinic dehydrogenase, which is interpreted as due to a more heterogeneous intracellular localization of this enzyme as compared to the oxidative enzymes.

Barth and Jaeger (1947) demonstrated that apyrases with different pH activity curves are present in several protein fractions of the amphibian embryo. A similar fractionation of proteins and measurements of the associated apyrases were not possible on *Ciona* blastomeres, due to the minute amounts of material available for analyses. The measurements made therefore represent total apyrase activities of the homogenates and, although there may be qualitatively different apyrases, the similarity of the pH-activity curves for anterior and posterior blastomeres indicates that segregation of this enzyme at the second cleavage is mainly quantitative.

Although acid phosphatase has been found to be intracellularly localized in small mitochondria of adult cells (Appelmans *et al.*, 1955), in the present experiments the opposite distribution of oxidative enzymes and acid phosphatase suggests a non-mitochondrial localization of the latter. It is possible that the localization of acid phosphatase may be a consequence of an unequal distribution of mitochondria. Thus a 2:1 distribution of mitochondria, as indicated by enzyme analyses, might cause displacement of a non-mitochondrial constituent into anterior cells.

Without information on the intracellular localization of ribonucleic acid in the ascidian egg, little can be said regarding the higher ribonucleic acid content of the

posterior cells. A factor other than mitochondrial segregation may be involved since mitochondria have a low content of ribonucleic acid (Schneider and Hogeboom, 1951, 1956).

The results are not due to volume differences since extensive measurements, discussed previously, of diameters and total protein in the two types of cells failed to demonstrate any significant differences in volumes. A differential solubility of ribonucleic acid in anterior and posterior blastomeres might lead to erroneous results; however, there is no indication of this, in that continuous extraction for four days with perchloric acid did not change the ratio of amounts extracted.

These quantitative analyses do not, of course, give any information as to the significance of the chemical differences in subsequent differentiation. A few preliminary experiments of rearing embryos in graded concentrations of KCN failed to show, by visual observation of whole embryos, any obvious differential effects on differentiation of anterior and posterior cells. Recently, however, Reverberi (1957b) has shown specific effects of sodium azide, malonate, and selenite on differentiation of the musculature of ascidian larvae, an effect presumably due to blocking activities of mitochondrial enzymes. Previously Ries (1939), by displacement of cytoplasmic areas with centrifugation, had concluded that the presence of the oxidative enzymes was essential for muscle differentiation.

Although localization of oxidases may be of significance in subsequent differentiation of ascidian embryos and several other mosaic forms (*Tubifex,* Lehmann and Wahli, 1954; *Nereis,* Reverberi and Pitotti, 1940; *Myzostoma,* Pitotti, 1947), this is not a common process during cleavage of all mosaic eggs.

Quantitative measurements of cytochrome oxidase in AB and CD blastomeres of *Mytilus edulis* indicated no unequal distribution of the enzyme in this mosaic egg (Berg, unpublished). First-cleavage blastomeres were isolated by previously described methods (Berg, 1950) and cytochrome oxidase measured by a microspectrophotometric method (Berg, 1956). Twelve paired measurements of the enzyme in homogenates of isolated blastomeres gave an average ratio of enzyme activity for the two types of cells (AB/CD) of $0.98 \pm 0.03$ after correction for volume differences.

Furthermore cytochemical tests for oxidative enzymes failed to reveal segregation of these enzymes during early cleavage of *Sabellaria* (Raven *et al.,* 1950), *Chaetopterus* and *Pomatocerus* (Ries, 1937), *Hydroides* (Reverberi and Pitotti, 1940) and *Limnaea* (Raven, 1946).

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## Summary

Anterior and posterior blastomeres of the four-cell stage of *Ciona* were separated for quantitative microchemical analyses of succinic dehydrogenase, apyrase, acid and alkaline phosphatases, and ribonucleic acid. Larger amounts of succinic dehydrogenase, apyrase and ribonucleic acid were found in homogenates of posterior cells whereas acid phosphatase activity was higher in anterior cells. The pH-activity curves of apyrases from anterior and posterior cells are similar, indicating a quantitative segregation of this enzyme. The unequal distribution of succinic dehydrogenase, apyrase and, possibly indirectly, acid phosphatase, is probably the result of a segregation of mitochondria.

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